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Viral Heat Resistance and Infectious Ribonucleic Acid

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High-titer suspensions of poliovirus 1 and coxsackievirus B-2 were shown to contain a heat-resistant fraction when heated for 65 min at temperatures ranging from 56 to 70°C. The addition of ribonuclease to the heated suspensions eliminated plaque production in the cell cultures, indicating that the resistant fraction was infectious ribonucleic acid that had been liberated from ruptured viruses during the heating process.

Although most viruses are usually heat sensitive, a number of investigators have reported that some viruses can survive temperatures ranging from 60 to 98°C (3-5, 8, 12, 14, 15). In addition, recoveries have been made of virus strains that pass their heat resistance characteristics to their progeny (2, 6, 11). Viruses apparently resistant to some heat processes were also detected in this laboratory when high-titer suspensions were studied. However, no survivors were noted when virus suspensions containing $<10^4$ plaque-forming units (PFU)/ml were heated at 60°C for at least 30 min (17).

To obtain more information on the heat-resistant virus fraction, a number of high-titer virus suspensions were heated at various times and temperatures. Survivors were passed in cell culture and reheated at the same thermal process. No apparent increase in thermal resistance was noted. Other virus suspensions were heat processed, and heated replicates of tubes showing resistant fractions were reheated. No difference in the number of survivors was observed.

A series of experiments was initiated to determine whether the heat-resistant fraction was due to the resistant, intact viruses, to surface-denatured particles containing ribonucleic acid (RNA), or to liberated infectious RNA.

MATERIALS AND METHODS

Virus preparations. Poliovirus 1 (ATCC VR-59) and coxsackievirus B-2 (ATCC VR 29) were propagated at 36°C in Buffalo green monkey kidney cells grown in media containing 1 part of L-15, 3 parts of minimum essential medium, and 10% fetal bovine serum (9, 16). The bottle cultures were frozen and thawed three times, and the fluid suspensions were centrifuged at 5,000 × *g* for 15 min. The supernatants were removed and pooled. The virus suspension was titrated and distributed into about 100 10-ml ampoules, which were then flame sealed. The suspensions were heat processed immediately or frozen at −80°C until used.

Thermal process. The sealed ampoules were placed in racks and submerged for varying times and temperatures in a constant-temperature water bath that contained an automatic agitator (19). Usually the suspensions were processed at 60°C for 65 min. After the heat treatment, the ampoules were immediately cooled in an ice bath and tested, or they were stored at −80°C until used.

Agar overlay procedure. A plaque-forming assay system using Buffalo green monkey kidney monolayers (45 cm^2) in 6-ounce (0.17-liter) prescription bottles was used to monitor nucleic acid and virus plaques (9, 14). The diethylaminoethyl (DEAE)-dextran (molecular weight, 2×10^6) concentration was increased from 0.1 to 0.7 g/liter.

Reagents. The ribonuclease (RNase A; GIBCO Diagnostics) and deoxyribonuclease (DNase; Sigma) were used at concentrations of 100 μg/ml. Some RNase activity was noted in the DNase when tested against RNA. The immune poliovirus horse serum, type 1, was obtained from the Biologics Section of the Communicable Disease Center. The hyperimmune antiserum, coxsackievirus type B-2, was purchased from Microbiological Associates. Both antisera were diluted 1/100. Normal horse and rabbit sera were used as controls.

Poliovirus 1 RNA, prepared by phenol extraction procedures, was used to monitor the plaque-forming system and to determine the effectiveness of the RNase used in the study. The RNase completely eliminated plaque formation by RNA (Table 1). The effect of antiserum on RNA was variable, with an average of ~40% reduction in the nine experiments studied.

Studies to maximize plaque formation by RNA. A review of the literature showed that two media ingredients, $MgCl_2$ and DEAE-dextran, had been used successfully for increasing RNA plaque formation (18, 20). Increasing the concentration of $MgCl_2$ above that normally used in our agar overlay system (5 g/liter) caused lysis of the cell cultures. Increasing the DEAE-dextran concentration from 0.1 to 0.7 g/liter increased the number of coxsackievirus B-2 plaques from an average of 3 to 31 PFU/ml. The 0.7-g/liter concentration of DEAE-dextran was not toxic to the cell cultures and was subsequently used in

TABLE 1. *Effect of antiserum and RNase on poliovirus RNA*

Initial titer (PFU/ml)[a]	RNA + antiserum (PFU/ml)	RNA + RNase (PFU/ml)
700	200	0
300	200	0
500	400	0
200	100	0
450	350	0
100	27.5	0
100	22	0
175	10	0
300	300	0

[a] Average of four replicate bottle cultures.

the agar overlay media. The increase in DEAE-dextran did not produce increased plaque numbers from dilutions of unheated suspensions.

Additional experiments were performed to determine whether the heated virus suspension should be diluted in the $MgCl_2$–DEAE-dextran solution before inoculation onto the cell sheets and whether the cell sheets should be exposed to the $MgCl_2$–DEAE-dextran solutions for varying time periods before inoculation. The 0.7-g/liter DEAE-dextran was found to be toxic to the cell cultures, and the concentration had to be reduced to 0.5 g/liter. Both procedures increased plaque formation and were used in subsequent studies. The cell sheets were exposed to the $MgCl_2$–DEAE-dextran solution for 1 to 3 h before inoculation, and the viruses were suspended in the $MgCl_2$–DEAE-dextran solution.

The heated virus suspensions contained considerable debris, and it was not known whether there would be interference between these materials and nucleic acid or virus attachment to the cell sheet. Attachment of viruses or RNA was extremely rapid, and washing or the addition of RNase or antiserum immediately after inoculation appeared to have no effect on plaque formation. However, as a safeguard, the inoculum was placed in contact with the cell sheet for 1 h at 36°C. The cell sheets were then washed three times with minimum essential medium.

RESULTS

Replicate virus suspensions were heated at 60°C for periods of up to 1,500 min and examined for survivors. The conclusion reached after examination of the results (Fig. 1) was that intact viruses would not survive such a thermal treatment and that the results probably indicated the presence in our cell culture system of either infectious nucleic acids or surface-denatured virus particles still containing RNA.

Low-level virus titers are inactivated quickly at relatively low temperatures. The literature indicates a two-step inactivation curve, the first associated with protein denaturation and the second probably related to nucleic acid inactivation. At higher temperatures a logarithmic death curve is noted. With high titers (>4.5 logs)

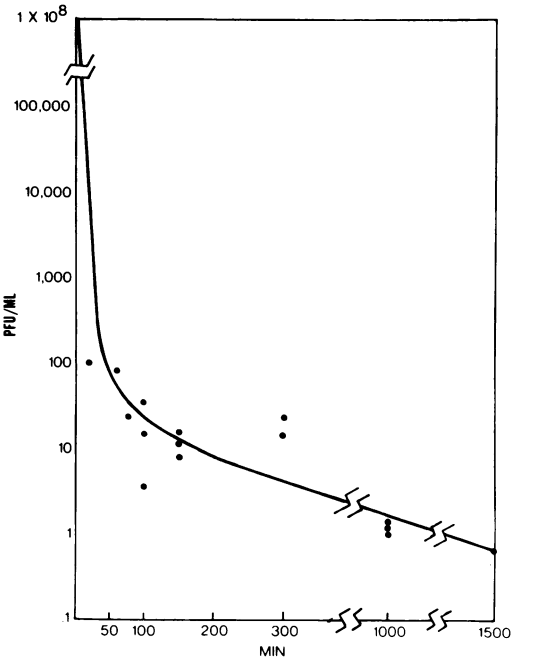

FIG. 1. *Thermal inactivation plot demonstrating apparent poliovirus 1 survivors for up to 1,500 min at 60°C.*

occasional survivors are detected. Most investigators studying virus thermal inactivation rates did not look for surviving populations but extrapolated from a number of points on a curve. We believe that nucleic acid is always released when single-stranded RNA viruses are heated. The RNA has a low level of detectable infectivity when compared with infectious virus, for 100 to 10,000 times more nucleic acid is required to produce one detectable infectious unit. Therefore, when low-level virus preparations are heated no survivors can be demonstrated, for the amount of RNA produced is below detectable levels.

A number of poliovirus 1 suspensions containing 10^8 PFU/ml were heated for 65 min at 60°C. The results (Table 2) show that the number of survivors or PFU in the suspensions ranged from 6 to 73 PFU/ml. Addition of specific antiserum to the heated suspensions had little or no effect on the number of plaques produced, but addition of RNase generally eliminated plaque production. Periodically a plaque was detected in the overlay system. Whether these plaques were produced by RNA or virus could not be determined. The reduction in plaque numbers by the antiserum in the RNA study was not observed when heated virus suspensions were processed.

We were concerned with a possible neutralizing effect of the viral debris in the heated sus-

pensions on antibody, so we diluted a coxsackievirus B-2 suspension and added antiserum or antiserum plus the heated virus suspension. No neutralizing effect on the antibody was noted.

A study was made on suspensions of coxsackievirus B-2 containing 7×10^7 PFU/ml that were heat processed at 60, 62.8, 65, and 70°C. The results (Table 3) show that plaques were detected after the addition of RNase in only one case. The addition of antiserum plus RNase produced the same results. The incorporation of antiserum, DNase, or normal horse or rabbit sera into the heated suspensions had little, if any, effect on the number of plaques observed, indicating that the plaques were probably produced by infectious nucleic acid.

TABLE 2. *Effect of antiserum and RNase on survivors of poliovirus 1 suspensions (10^8) heated for 65 min at 60°C*

Virus[a] titer (PFU/ml)[b]	Virus[a] + antiserum	Virus[a] + RNase (PFU/ml)	Virus[a] + antiserum + RNase (PFU/ml)	Virus[a] + horse serum (PFU/ml)
13.5	16.5	0.5	0.5	16.5
15.5	31.0	0	1.5	19.0
34.0	35.0	0	ND[c]	27.0
21.0	16.0	0	1	ND
6.0	7.0	0	ND	4.0
9.0	7.0	4	ND	4.0
13.5	16.5	0.5	ND	16.5
37.0	35.0	0	ND	23.0
23.0	20.0	0	0	ND
35.5	35.0	0	ND	25.0
22.0	18.0	0	0	22.5
73.0	53.0	1.3	ND	ND
19.3	18.3	1.0	ND	ND
47.25	ND	ND	ND	ND

[a] Heated-surviving fraction.
[b] Average of four replicate bottle cultures.
[c] ND, Not done.

We were informed that the DNase used in this study was contaminated with RNase. To determine if we could detect this activity, we removed from the freezer a number of ampoules of heat-processed poliovirus 1 suspensions that had been stored for more than 1 year at −70°C. Varying dilutions of RNase and DNase were added to the suspensions, and the dilutions were processed in cell culture. The data shown in Table 4 indicated DNase activity at the 100-μg level, which demonstrated the presence of some RNase contamination. The RNase was active to the 1-μg level. Examination of the data outlined in Table 3 showed no detectable RNase activity in the DNase preparations when tested on the heated virus suspensions. These studies were made on unfrozen preparations. We have noted, as have other investigators, that the detectable activity of RNA is gradually lost during frozen storage. Possibly the RNA activity was reduced during storage of the heated suspension to a level suitable for detection of the RNase contaminant of the DNase. This contamination is indicated in Table 5, where the enzymatic activity of RNase and DNase on poliovirus 1 RNA is tabulated.

The data demonstrated the effect of both antisera and RNase on the surviving fractions. This was especially true at the 5- and 10-min periods. We picked the 52°C data because little effect by the antiserum could be observed at any of the higher temperatures used. At lower temperatures the numbers of virus survivors were too high to demonstrate RNA activity.

TABLE 3. *Effect of antiserum, RNase, and DNase on survivors of coxsackievirus B-2 suspensions (7×10^7) heated for 65 min at 60, 62.8, 65, and 70°C*

Temp (°C)	Virus[a] titer (PFU/ml)[b]	Virus[a] + antiserum (PFU/ml)	Virus[a] + RNase (PFU/ml)	Virus[a] + antiserum + RNase (PFU/ml)	Virus[a] + DNase (PFU/ml)	Virus[a] + rabbit serum (PFU/ml)
60	13	13	0	0	10	9
	11	11	0	0	13	4
	3.5	5.5	0	0	7.5	3
	3.5	5.0	0	0	6.0	3
62.8[c]	3.75	6.5	0	0.25	6.5	6.25
65	8	17	0	0	25	13
	26	9	0	0	22	13
	12	15	0.5	0.5	14	14
	9	5.5	0	0	13	5
70	11	9	0	0	13	9
	14	16	0	0	20	14
	17	16.5	0	0	12	6.5
	5.5	8	0	0	11.5	7

[a] Heated-surviving fraction.
[b] Average of four replicate bottle cultures.
[c] Input virus titer, 5×10^6 PFU/ml.

TABLE 4. *Effect of RNase and DNase on the surviving heat-resistant fraction[a] of a poliovirus 1 suspension heated at 60°C for 60 min*

Enzyme	μg/ml	PFU/ml[b]
RNase	100.0	0.7
	10.0	0.3
	1.0	2.0
	0.1	7.0
	0.01	12.7
DNase	100	1.7
	10	12.3
	1	16.0
	0.1	9.0
	0.01	11.0

[a] ~12.3 PFU/ml (average of four bottle cultures).
[b] Average PFU of four bottle cultures.

TABLE 5. *Effect of RNase and DNase on poliovirus/RNA[a]*

Enzyme	μg/ml	PFU/ml
RNase	100.0	0
	10.0	
	1.0	0
	0.1	0
DNase	100.0	0
	10.0	0
	1.0	10
	0.1	31

[a] Control RNA + minimum essential medium, ~50 PFU/ml.

The liberation of nucleic acid from viruses during thermal processing was described by Bachrach (1), who advocated this procedure as a means of recovering nucleic acid. A graphic representation of nucleic acid liberation from poliovirus 1 was shown by McGregor and Mayor (10). Long strands of nucleoprotein were demonstrated in electron micrographs when virus suspensions were heated at 45°C for 45 s to 5 min.

A number of plaques that were recovered from the heat-treated samples were picked and passed in Buffalo green monkey kidney cells. Neutralization tests showed that the progeny were either poliovirus 1 or coxsackievirus B-2. A useful practical application of this procedure would be the purification of virus stocks contaminated with mycoplasma, fungi, or bacteria that were sensitive to the heat treatment or mixed virus stocks where the contaminating viruses were present in low titer.

Experiments were also performed in which RNase was added to the virus suspensions and then heat processed. The RNase was not destroyed and effectively eliminated plaque production in the heated preparations. RNase was added to a series of dilutions of unheated virus suspensions, and plaque counts were compared with controls to determine whether the enzyme affected the intact viruses or if free nucleic acid could be detected. The RNase had no apparent effect on the unheated viruses. In addition, RNA was heated for 65 min at 60°C. There was a 60 to 90% reduction in the number of plaques produced after the heat treatment. Therefore, the nucleic acid produced during the heat processing of the virus suspensions must also be partially inactivated during the 65-min treatment.

A varying number of infectious units survived in the heat-treated suspensions. If cell lysates or enzymes adversely affected the heated virus particles or RNA, this effect would have already occurred and the detected plaque-forming agents are survivors of such postulated adverse reactions. Such heat-resistant surviving fractions are seen in the first columns of Tables 2, 3, and 6.

In an attempt to determine the effect of cell lysate on RNA, the data in Table 7 were compiled. The lysate appeared to inactivate RNA up to a dilution of 1/1,000. A comparison of the effect of heated and unheated Buffalo green monkey kidney cell lysate on the poliovirus heat-processed survivors was determined as shown in Table 8. Both the heated and unheated lysate had some effect on the number of detectable survivors but did not eliminate them completely. The heated lysate appeared to be slightly less effective than the nonheated lysate.

In Table 9 RNA was added to the heated virus suspension in an attempt to detect the presence of RNase that might have been in the cell lysate when the virus suspensions were prepared. The number of plaques detected in the RNA suspensions exceeded the number detected in the same suspensions without added RNA, indicating little, if any, RNase activity. It is interesting that when dilutions are made of the heated suspension, we always detect more plaques than expected.

Koch reported the presence of denatured poliovirus 1 in suspensions that had been heated at 54°C (7). These particles were reported to be incorporated into cells in the presence of 1 M NaCl, pH 8.0, but they were not incorporated when suspended in saline. The efficiency of uptake was 1 in 10^4 particles. The denatured particles probably could not attach to the cells because of surface structural changes, but they could be engulfed by the cell. NaF has been used by other investigators to inhibit phagocytosis (13). Therefore, NaF was added to culture media and to the virus suspensions to attempt to inhibit

TABLE 6. *Effect of antiserum, RNase, and DNase on survivors of coxsackievirus B-2 suspensions (3.5×10^7) heated at 52°C*

Time at 52°C (min)	Virus[a] titer (PFU/ml)	Virus[a] antiserum (PFU/ml)	Virus[a] RNase (PFU/ml)	Virus[a] + antiserum + RNase (PFU/ml)	Virus[a] DNase (PFU/ml)	Virus[a] + rabbit serum (PFU/ml)
5	6,850	150	2,400	0	3,250	3,800
10	300	100	450	0	600	600
20	40	30	20	0	30	50
30	20	30	10	10	ND[c]	25
40	30	15	0	0	30	20
50	3	18	3	1	9	17
60	10	11	3	4	15	17
60	27	23	1	ND	ND	ND
60[b]	200	170	21	ND	ND	ND

[a] Heated-surviving fraction.
[b] Input virus titer, 2.9×10^8.
[c] ND, Not done.

TABLE 7. *Effect of Buffalo green monkey kidney cell lysate (not virus infected) on RNA*

Sample	PFU/ml
RNA + minimum essential medium	67
RNA + cell lysate	0
RNA + cell lysate (10^{-1})	0
RNA + cell lysate (10^{-2})	0
RNA + cell lysate (10^{-3})	15
RNA + cell lysate (10^{-4})	35

TABLE 8. *Effect of heated and unheated Buffalo green monkey kidney (BGM) cell lysate (not virus infected) on poliovirus 1 suspensions heated at 60°C for 65 min*

Sample	PFU/ml
Poliovirus 1 heated at 60°C for 65 min	12.3
Poliovirus 1 heated at 60°C for 65 min + BGM cell lysate unheated	1.5
Poliovirus 1 heated at 60°C for 65 min + BGM cell lysate heated at 60°C for 65 min	4.0

TABLE 9. *Effect of heated virus cell lysate on RNA*

Sample	Avg PFU/ml
RNA + PBS	21.0
RNA + P1[a] suspension heated at 60°C for 65 min	52.0
RNA + P1 heated at 60°C for 65 min, diluted (10^{-1})	22.5
RNA + P1 heated at 60°C for 65 min, diluted (10^{-2})	12.5
RNA + P1 heated at 60°C for 65 min, diluted (10^{-3})	21.5
P1 suspension heated at 60°C for 65 min + PBS[b]	36.0
P1 suspension heated at 60°C for 65 min + PBS (10^{-1})	15.0
P1 suspension heated at 60°C for 65 min + PBS (10^{-2})	4.5
P1 suspension heated at 60°C for 65 min + PBS (10^{-3})	0.5

[a] P1, Poliovirus 1.
[b] PBS, Phosphate-buffered saline.

the phagocytic activity of the cells. No apparent difference was noted between the number of plaques produced in the NaF-treated and the untreated cells when heated suspensions were processed, indicating that denatured particles probably were not associated with the heat-resistant fraction.

Portions of the poliovirus 1 and coxsackievirus B-2 suspensions that had been heated at 60°C for 65 min were dialyzed against polyethylene glycol. The concentrated suspensions were layered onto cesium chloride gradients and centrifuged in an SW65 Ti Spinco rotor for 20 h at 50,000 rpm. Fractions were removed, and the number of plaques produced by each fraction was determined. The majority of the plaques were produced from the fractions between 1.57 and 1.69 g/cm^3, which are in the density range of RNA. Aliquots of all fractions were exposed to RNase and processed in cell culture. No plaques were produced.

The presence of free nucleic acid might not be detected in some cell culture systems if the osmotic concentration or the virus titer of the medium was low. Relatively high titers of viruses ($>10^{4.5}$) were heated before the nucleic acid was detected in our system. Considerable undetected nucleic acid must still have been present in the fluid suspensions. The live animal or human could be a more sensitive detection system, and infection might result from injection or consumption of heated material that had shown no activity in cell culture.

DISCUSSION

The addition of either poliovirus 1 or coxsackievirus B-2 antisera to the heated suspensions

had little or no effect on the heat-resistant fraction. No inhibition in plaque production was observed when NaF was added to the cell cultures to inhibit engulfment of damaged particles, or when DNase or normal serum was added to the heated preparations. The majority of the plaque-producing fractions recovered from cesium chloride gradients were in the density range of RNA. Aliquots of these fractions failed to produce plaques when treated with RNase. The only substance tested that eliminated plaque production in the cell cultures was RNase. Therefore, infectious RNA, which was liberated from damaged viruses during the heating process, was the resistant fraction in the heat-treated virus suspensions. Viruses recovered from the plaques produced from the heated suspensions were shown by neutralization tests to be either poliovirus 1 or coxsackievirus B-2.

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